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Cinnamic Acid, an Autoinducer of Its Own Biosynthesis, Is Processed via Hca Enzymes in *Photorhabdus luminescens*▽

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***Photorhabdus luminescens*, an entomopathogenic bacterium and nematode symbiont, has homologues of the Hca and Mhp enzymes. In *Escherichia coli*, these enzymes catalyze the degradation of the aromatic compounds 3-phenylpropionate (3PP) and cinnamic acid (CA) and allow the use of 3PP as sole carbon source. *P. luminescens* is not able to use 3PP and CA as sole carbon sources but can degrade them. Hca dioxygenase is involved in this degradation pathway. *P. luminescens* synthesizes CA from phenylalanine via a phenylalanine ammonia-lyase (PAL) and degrades it via the not-yet-characterized biosynthetic pathway of 3,5-dihydroxy-4-isopropylstilbene (ST) antibiotic. CA induces its own synthesis by enhancing the expression of the *stlA* gene that codes for PAL. *P. luminescens* bacteria release endogenous CA into the medium at the end of exponential growth and then consume it. Hca dioxygenase is involved in the consumption of endogenous CA but is not required for ST production. This suggests that CA is consumed via at least two separate pathways in *P. luminescens*: the biosynthesis of ST and a pathway involving the Hca and Mhp enzymes.**

Cinnamic acid (CA) and its derivatives are secondary metabolites with antioxidant and antibacterial activities produced by plants in response to stressful conditions, such as infections or wounding (21). These compounds are particularly interesting in a wide range of applications. CA is used as a flavoring agent in baked goods, sweets, ice cream, beverages, and chewing gum (33). CA inhibits the growth of several bacteria (7), in particular *Escherichia coli* O157:H7 (40), and is an active compound in medicinal plants with anti-*Helicobacter pylori* (1) or antituberculosis activity (2). It also enhances the activity of drugs like isoniazid or rifampin against *Mycobacterium tuberculosis* (30). CA derivatives also have additional activities: *o*-methyl cinnamamide inhibits the invasion and metastasis of human malignant melanoma (39), cinnamaldehyde is antimutagenic in mammalian cells (20), and flavonoid compounds with a CA backbone have cancer chemopreventive, antioxidant, and antiasthmatic activities (18). In plants, the synthesis of CA from phenylalanine is catalyzed by phenylalanine ammonia-lyase (PAL; EC 4.3.1.5). In industry, CA is produced by chemical syntheses. As chemical syntheses involve large amounts of solvents, there is a growing interest in developing alternative, environmentally friendly procedures. Nijkamp et al. constructed a *Pseudomonas putida* strain expressing the PAL enzyme from *Rhodosporidium toruloides* and secreting CA at a concentration of 5 mM in the medium (27). PAL homologs are found in various plants, fungi, and yeasts but in only two prokaryotes, *Streptomyces maritimus* (43) and *Photorhabdus luminescens* (41).

The bacterium *P. luminescens* (*Enterobacteriaceae*) is an insect pathogen and a nematode symbiont. After entering the insect host, the nematode, at the infective juvenile stage, releases its bacterial symbionts into the insect hemocoel, in which bacteria proliferate rapidly (10, 13). The insect host succumbs within 24 to 48 h of bacterial infection to bacterial exo- and endotoxins which are produced as the bacteria multiply (3, 4, 17). The bacteria also produce antibiotics that inhibit the growth of competing microorganisms in the insect cadaver (11, 25, 32). CA is a precursor of one of these antibiotics, 3,5-dihydroxy-4-isopropylstilbene (ST). ST is also an inhibitor of melanization, which is a part of the insect immune response (15). The first step of the ST biosynthesis pathway is the deamination of phenylalanine, catalyzed by PAL and resulting in CA (Fig. 1A) (41). The subsequent steps are unknown.

The *P. luminescens* strain TT01 genome sequence shows that the bacterium has homologs of the *hcaR*, *-E*, *-F*, *-C*, *-B*, and *-D* genes present in *Escherichia coli* (14). The *E. coli hcaE*, *-F*, *-C*, *-B*, and *-D* operon encodes enzymes involved in the first two steps of CA and 3-phenylpropionate (3PP) catabolism (6, 12) (Fig. 1B). The *hca* operon and *mhp* genes allow *E. coli* to grow on 3PP as sole carbon source (12). Introduction of the *E. coli hca* and *mhp* genes into *Salmonella enterica* serovar Typhimurium allows the growth of *S. enterica* on CA (12). In addition, *hca* operon expression in *E. coli* (12) is positively regulated by HcaR, a member of the LysR family of regulatory proteins (36). This is also the case in *P. luminescens* (8). In *P. luminescens*, HcaR is also involved in the oxidative stress response, toxemia, and virulence (8). The effects of HcaR on virulence are not related to its role in the control of *hca* operon expression, as disruption of *hcaE* does not affect virulence or toxemia (8).

In the work presented in this report, the involvement of the Hca enzyme in CA utilization and ST synthesis in *P. luminescens* was investigated.

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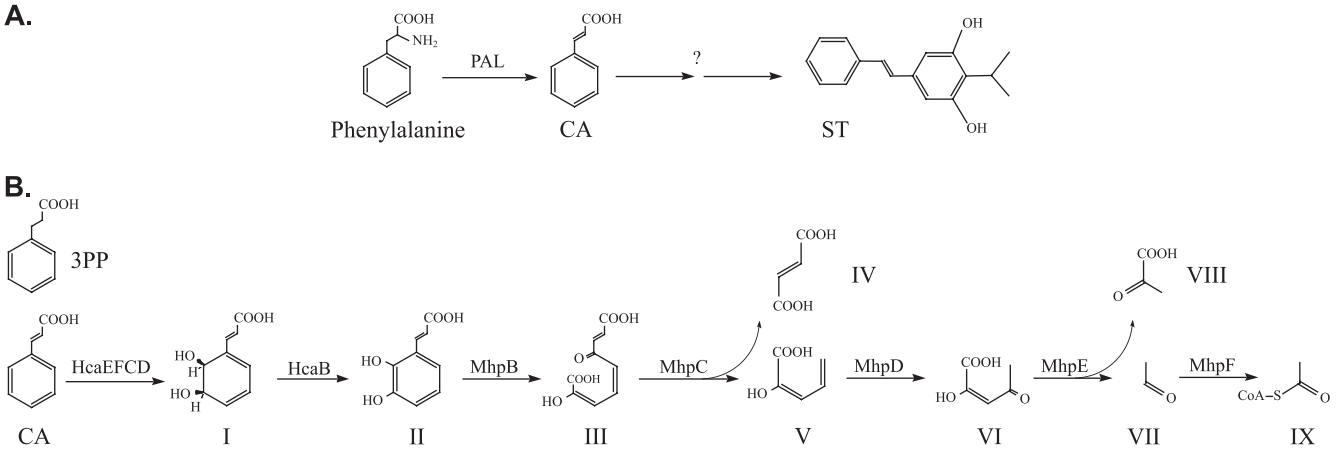

FIG. 1. Utilization of CA by *P. luminescens* (A) and *E. coli* (B). (A) Biosynthetic pathway of ST in *P. luminescens* (41). CA, formed from phenylalanine, is a precursor of ST. (B) Pathway for the degradation of CA by *E. coli* (12). Metabolites are as follows: *cis*-3-(3-carboxyethenyl)-3,5-cyclohexadiene-1,2-diol (I), 2,3-dihydroxycinnamic acid (II), 2-hydroxy-6-ketononatrienedioate (III), fumarate (IV), 2-keto-4-pentotenoic acid (V), 4-hydroxy-2-ketovaleric acid (VI), pyruvic acid (VII), acetaldehyde (VIII), and acetyl-CoA (IX). 3PP degradation is catalyzed by the same enzymes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *P. luminescens* strains used in this study were TT01R (a spontaneous rifampin-resistant mutant of *P. luminescens* subsp. *laumondii* TT01) (8), used as the wild-type reference in all experiments, its *hcaE* derivative strain TT01Δ*hcaE* (8), and its *hcaR* derivative strain TT01Δ*hcaR* (8). *P. luminescens* strains were routinely grown under aerobic conditions at 30°C in Luria-Bertani (LB) or in Schneider medium (Bio-Whittaker). If indicated, 3PP (Sigma) and CA (Sigma) were added to a concentration of 1.3 mM each. LB medium was solidified with 1.5% Difco agar as required.

RT-PCR and real-time quantitative RT-PCR. To determine whether *hcaEFCD*-plu2208-plu2209 is an operon, reverse transcription-PCR (RT-PCR) was performed using primers designed to amplify three intergenic regions of the *hca* locus. Strain TT01R was grown in Schneider medium supplemented with 0.5 mM cyclic AMP, in the presence of 1.3 mM 3PP. Bacteria were harvested at the beginning of the stationary phase (optical density at 600 nm of 9). Total RNA was prepared as previously described, using the Trizol method (11). Single-step RT-PCR was performed with 250 ng of total RNA using an AccessQuick RT-PCR system kit (Promega) according to the manufacturer's instructions. The reaction mixture was incubated at 48°C for 45 min and was then subjected to PCR (40 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 68°C). We confirmed that none of the RNA samples was contaminated with DNA by performing 40 cycles of PCR with 250 ng of total RNA as the template. The RT-PCR products were analyzed by electrophoresis on 2% agarose gels, using UltraPure agarose (Invitrogen Corp.). Primers were used at a concentration of 1 μM (see Table 1 for primer sequences).

Quantitative RT-PCR was performed as previously described (22). The quantity of cDNA for each gene studied was normalized to the quantity of *udp* cDNA in each sample; *udp* was considered to represent a stably expressed housekeeping gene. To check for contaminating chromosomal DNA, each sample was tested in control reactions without reverse transcriptase. The relative change in gene expression was recorded as the ratio of normalized target concentrations (threshold cycle) (26).

PAL enzyme assay. PAL activity was measured as previously described (23, 41). Bacteria were grown in LB; 40-ml amounts were collected at various times and centrifuged. The cells were resuspended in 2 ml 0.1 M sodium borate buffer (pH 8.8) and sonicated. The protein concentrations in the extracts were determined by using a Bradford reagent assay (Sigma) using bovine serum albumin as the standard. The PAL activity was measured by adding 600 μg of protein into a 20 mM *l*-phenylalanine solution prepared in 0.1 M sodium borate buffer (pH 8.8). The final reaction mixture volume was 1 ml. The reaction tubes were incubated at 30°C, and the production of CA was detected by measuring the increase in A_{290}.

HPLC analysis. All samples were filtered through filters with 0.45-μm pores before injection onto the high-pressure liquid chromatography (HPLC) column. 3PP and CA were detected with an Agilent 1100 HPLC system using a Lichrosphere 5 RP-8 column (150 × 4.6 mm). We used the following program: a linear gradient from 0 to 100% acetonitrile in water supplemented with 0.1% trifluoroacetic acid over 20 min; this was followed by the isocratic mode (100% acetonitrile) for 7 min. The mobile phase was delivered at 1 ml/min. 3PP was detected at 210 nm with a 13.31-min retention time. CA was detected at 280 nm with a 13.45-min retention time.

For quantitative analyses, a standard curve of CA was established. The following equation was obtained for those standard solutions with concentrations of CA from 0.013 mM to 1.3 mM: $C = 7.200\ A - 0.021$, where A is the area recorded (recorded units/100,000, with detection at 280 nm) and C is the concentration of CA (mM) in the 20-μl sample injected into the HPLC column. With a total of five points, the coefficient of regression was 0.9981.

Purification and identification of CA. Strain TT01Δ*hcaE* was grown in 100 ml LB for 48 h. The culture supernatant was dried and eluted through a silica gel column using a solvent made of dichloromethane:methanol (99:1, progressively enriched until 94:6). The presence of putative CA was checked by analyzing elution fractions on a thin-layer chromatography plate (aluminum-backed silica gel 60 F_{254}), using a solvent of dichloromethane:methanol (95:5), which was visualized by UV at 254 nm. Elution fractions containing pure putative CA were then pooled together and evaporated under vacuum. The resulting product was resuspended in dimethyl sulfoxide.

The proton nuclear magnetic resonance (NMR) spectrum of this compound in dimethyl sulfoxide was recorded on a Bruker Avance spectrometer (400 MHz). The analytical results (thin-layer chromatography and NMR) were in agreement with those for commercially available CA.

RESULTS

***Photorhabdus luminescens* has homologs of genes involved in the known phenylpropionate/cinnamate catabolic pathway.** In *E. coli* K-12, catabolism of the aromatic compounds 3PP and CA involves the same enzymes (12) (Fig. 1B). The first two steps, catalyzed by the dioxygenase HcaEFCD and the dehydrogenase HcaB, activate the aromatic ring by forming a dihydroxy derivative (compound II in Fig. 1B). Ring cleavage is then catalyzed by MhpB (Fig. 1B). The subsequent steps are catalyzed by other Mhp enzymes, which ultimately break this compound down into Krebs cycle intermediates: fumarate (compound IV), pyruvate (compound VII), and acetyl coenzyme A (acetyl-CoA) (compound IX). The *E. coli* genes involved in 3PP/CA catabolism are located in two clusters: the *hca* cluster, encoding proteins involved in the two first steps,

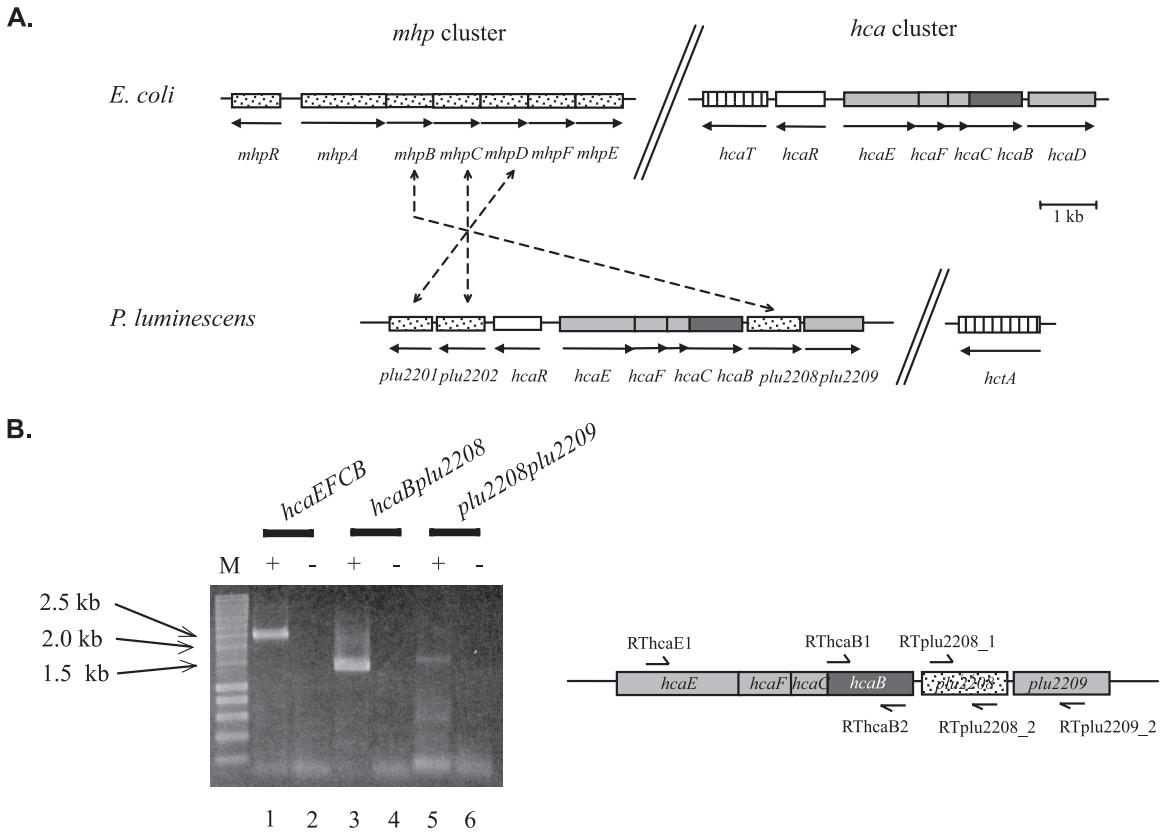

FIG. 2. (A) Genetic organization of the *hca* and *mhp* genes in the *E. coli* and *P. luminescens* chromosomes. Solid arrows indicate the direction of gene transcription. Dotted arrows indicate homology between plu2201 and *mhpD*, plu2202 and *mhpC*, and plu2208 and *mhpB*. For *hca* genes, genes with similar shadings encode subunits of the same protein. (B) Detection of the *hcaEFCB*-plu2208-plu2209 transcript by RT-PCR. RT-PCR products were analyzed by electrophoresis in 2% agarose gels. RT-PCR was performed with (+) and without (−) reverse transcriptase. Lanes 1 and 2, *hcaEFCB* transcript (expected size, 2.5 kb), produced with primers RThcaE1 and RThcaB2; lanes 3 and 4, *hcaB*-plu2208 transcript (expected size, 1.4 kb), produced with primers RThcaB1 and RTplu2208_2; lanes 5 and 6, plu2208-plu2209 transcript (expected size, 1.6 kb), produced with primers RTplu2208_1 and RTplu2209_2 (see Table 1 for primer sequences); lane M, SmartLadder size marker. Molecular sizes are indicated on the left.

and the *mhp* cluster, encoding proteins involved in the following steps (Fig. 2A). *P. luminescens* has homologs of the HcaR, -E, -F, -B, -C, and -D proteins, with sequence identities of 47%, 81%, 70%, 62%, and 63%, respectively. The MhpB, MhpC, and MhpD homologs in *P. luminescens* are Plu2208, Plu2202, and Plu2201, respectively, with amino acid identities of 41% (e-value of 5e-73), 28% (e-value of 8e-20), and 37% (e-value of 2e-49), respectively. Their coding genes flank the *hcaR*, *-E*, *-F*, *-C*, and *-B* genes (Fig. 2A). No homolog of *mhpE* was found. Also, no homolog of *mhpF*, encoding an acetaldehyde dehydrogenase, was identified, but we did detect an *adhE* homolog (plu2496), encoding an acetaldehyde dehydrogenase (19). Remarkably, the genes involved in 3PP/CA catabolism are organized into two clusters in *E. coli*, but their counterparts in *P. luminescens* all map to a single cluster, with the exception of the *hcaT* homolog (putative transporter of 3PP), located outside the cluster. The *hcaR* gene, which presumably encodes the regulator of the putative operon, is transcribed divergently, as is the case for many LysR-type regulators (34) (Fig. 2A).

The *hca* operon of *P. luminescens* includes an additional gene, plu2208, not found in the *E. coli* operon (Fig. 2A). Plu2208 is a homolog of MhpB, catalyzing the third step of 3PP/CA catabolism in *E. coli* (Fig. 1B). This led us to investigate whether *hcaE*, *hcaF*, *hcaC*, plu2208 (*mhpB* homolog), and plu2209 (*hcaD* homolog) were expressed as a single operon in *P. luminescens*. RT-PCRs were carried out with three pairs of primers (Table 1; Fig. 2B). Total RNA was extracted from the TT01R strain grown in Schneider medium supplemented with 1.3 mM 3PP to induce the expression of the putative *hca* operon and cyclic AMP to prevent the glucose-dependent repression of *hca* gene expression (38). An amplicon of the expected size was obtained in all cases: 2.5 kb for the *hcaEFCB* amplicon, 1.4 kb for the *hcaB*-plu2208 amplicon, and 1.6 kb for the plu2208-plu2209 amplicon (Fig. 2B). Thus, in *P. luminescens*, *hcaE*, *-F*, *-C*, and *-B* and plu2208 and plu2209 are expressed as an operon, whose homologs in *E. coli* are involved in the first three steps of 3PP/CA catabolism and result in the ring-cleavage product of 3PP/CA.

***Photorhabdus luminescens* is not able to use 3PP or CA as a sole carbon source, but degrades them.** The *hca* and *mhp* genes allow *E. coli* to grow on 3PP as the sole carbon source (6, 12). Whether this was also the case in *P. luminescens* was investigated. Bacteria TT01R did not grow in minimal medium containing 3PP. The introduction of the *E. coli hca* and *mhp* genes

TABLE 1. Nucleotide sequences of primers used in RT-PCR and quantitative RT-PCR experiments

Assay and gene	Primer(s)	Sequence(s)
RT-PCR		
hcaE	RThcaE1	5′-GCAAAGAGCAATGGGGACTTCAGG-3′
hcaB	RThcaB1, RThcaB2	5′-GGTAACGATATCTGTGTTATTCACG-3′, 5′-ATTGCACTGAGACTTTCT AATGACA-3′
plu2208	Rtplu2208_1, Rtplu2208_2	5′-GGAGTAAGAGCGAATGCAGTGGGGG-3′, 5′-GATTCAACGGAGCAA CGATATTCTC-3′
plu2209	Rtplu2209_2	5′-CTCTCTGAAGGCAAACATCCCCGGC-3′
Quantitative RT-PCR		
udp	udp5quan, udp3quan	5′-CGGGCTCAACCTGGGAATGG-3′, 5′-CTCCACATCTCTACGCATTTC-3′
hcaE	qRThcaE1, qRThcaE2	5′-ATTAATCGACGTTCCGTTGG-3′, 5′-GGGCTGTCGTATCCCAGTTA-3′
stlA	stlA5quan, stlA3quan	5′-TGTTTGCAAAGGTTGGTCTG-3′, 5′-GGAATTAAATCACCGCTTGC-3′
plu2201	qRTplu2201_1, qRTplu2201_2	5′-CGATTTTGGCATTCTCACCT-3′, 5′-ATTTCGATACAGGGCACGAC-3′
plu2202	qRTplu2202_1, qRTplu2202_2	5′-TATGTTGTCCGCAATGGAAA-3′, 5′-AAGACGCTGGCCTACTGAAA-3′

into *Salmonella enterica* serovar Typhimurium allows *S. enterica* growth on CA (12). The ability of *P. luminescens* to grow in minimal medium containing CA as the sole carbon source was tested. No growth was observed. This inability may be related to the fact that no *mhpE* and *mhpF* homologs, required for the two last steps of catabolism, were identified. However, *E. coli*, despite the presence of *mhpE* and *mhpF*, degrades CA but does not use it as sole carbon source (12).

The degradation of 3PP or CA by *P. luminescens* was investigated. The wild-type strain TT01R was grown in LB medium supplemented with 1.3 mM 3PP or 1.3 mM CA, and the 3PP and CA amounts in the culture supernatants were assayed by HPLC analysis. Both the 3PP and CA quantities rapidly decreased between 16 and 24 h of culture, during the transition from exponential growth to stationary phase (Fig. 3). The involvement of Hca enzymes in this process was investigated; thus, the degradation of 3PP and CA by the TT01Δ*hcaE* mutant strain grown in the presence of these compounds was monitored. The first gene of the *hca* operon, *hcaE*, encoding the alpha subunit of the Hca dioxygenase which catalyzes the first step of 3PP/CA catabolism in *E. coli*, was disrupted in this mutant (Fig. 1B). The amounts of 3PP and CA in culture supernatants of strain TT01Δ*hcaE* remained constant during growth from 0 to 80 h (Fig. 3B). Thus, the consumption of 3PP and CA requires the activity of the Hca dioxygenase. This result establishes that the first step of 3PP/CA degradation catalyzed by the Hca dioxygenase occurs in *P. luminescens*.

HcaE is involved in degradation of endogenous CA. Unlike most known enterobacteria, *P. luminescens* expresses a PAL enzyme, catalyzing CA formation from phenylalanine (41). CA was previously detected in bacterial extracts (5). The presence of CA in the culture supernatants of TT01R grown in LB medium was analyzed by HPLC. A compound was secreted by *P. luminescens* that accumulated after 16 h of growth (Fig. 4A). This compound eluted at the same position as CA, and it had a similar UV spectrum to CA (data not shown). After purification on silica gel, the identity of this compound was confirmed by proton NMR (data not shown). The concentration of CA released by TT01R into the culture supernatant increased at the end of the exponential growth phase, between 8 and 16 h of culture, reaching 0.2 mM, and then it rapidly decreased to 0.05 mM after 20 h (Fig. 4B). The CA concentration eventually stabilized at 0.01 mM (Fig. 4B). Thus, *P. luminescens* released a considerable amount of CA into the medium and then subsequently processed it.

HcaE is involved in the degradation of exogenous CA. To know whether HcaE is involved in the degradation of endog-

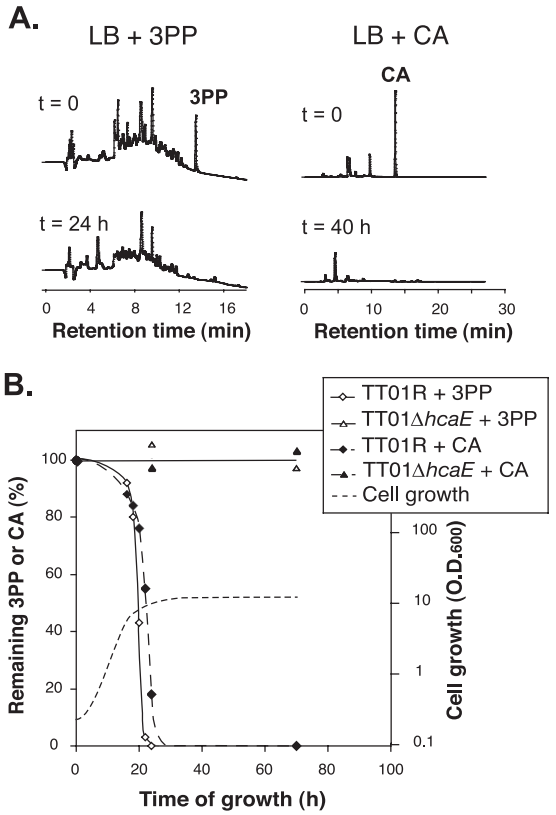

FIG. 3. Consumption of 3PP and CA by *P. luminescens*. The parent TT01R and mutant TT01Δ*hcaE* strains were grown in LB medium containing 1.3 mM 3PP or 1.3 mM CA. Culture supernatants were collected at various times and analyzed by HPLC. (A) Chromatograms of culture supernatants of TT01R, recorded with detection at 210 nm for 3PP-containing culture and 280 nm for CA-containing supernatants. (B) 3PP (open symbols) and CA (closed symbols) quantities remaining in culture supernatants, and cell densities (dotted line) of TT01R and TT01Δ*hcaE*. Quantities of 3PP and CA are expressed as percentage of area of corresponding peak compared with initial area. $O.D._{600}$, optical density at 600 nm.

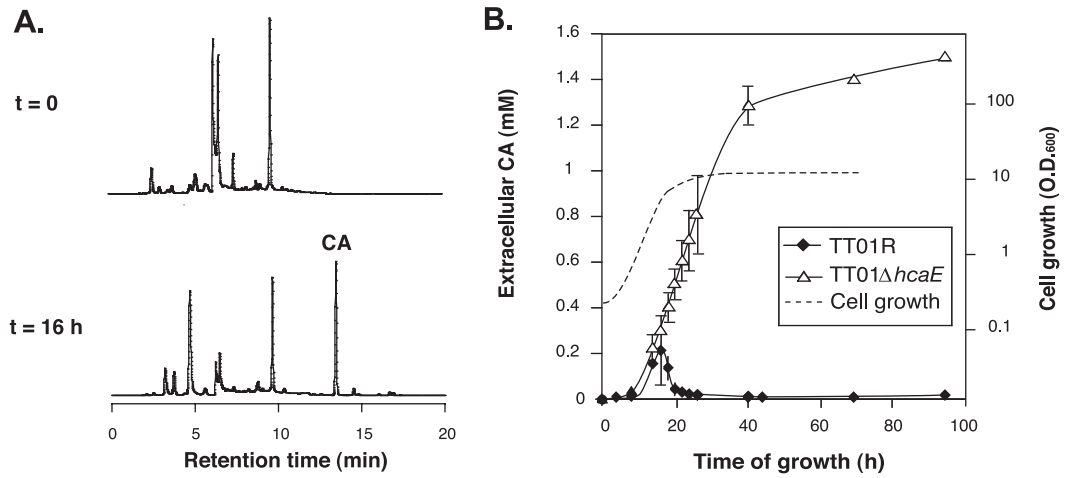

FIG. 4. Detection of CA in culture supernatants of *P. luminescens*. (A) HPLC chromatograms of LB medium (top) and of a 16-h-culture supernatant of TT01R grown in LB medium (bottom). Detection was performed at 280 nm. (B) The parent TT01R (diamonds) and the mutant TT01Δ*hcaE* (triangles) bacteria were grown in LB. Concentrations of CA in culture supernatant were determined by HPLC. Measurements are expressed as the means ± standard deviations of the results of two independent experiments. $O.D._{600}$, optical density at 600 nm.

enous CA, the culture supernatant of strain TT01Δ*hcaE* grown in LB was analyzed by HPLC. The concentration of CA increased at the end of the exponential growth phase, between 8 and 16 h of growth (Fig. 4B). After 16 h, in contrast to the results for the parental strain TT01R, the CA concentration kept increasing, reaching a value of 1.5 mM after 4 days of growth (Fig. 4B). Thus, the disruption of *hcaE* abolished the stationary-phase-dependent decrease of extracellular CA evidenced in the wild-type strain. This suggests that the Hca dioxygenase is involved in the catabolism of endogenous CA from TT01R culture supernatants.

Effect of *hcaE* disruption on ST production. In *P. luminescens*, CA is a precursor of the antibiotic ST (41). The pathway responsible for ST formation from CA is still unknown. Hca dioxygenase is involved in the consumption of exogenous and endogenous CA. As a consequence, Hca dioxygenase may catalyze the first step of the CA-to-ST pathway. The overlay method was used to test whether HcaE is required for ST formation (41). TT01R and TT01Δ*hcaE* mutant bacteria were spotted on LB agar and overlaid with soft agar containing *Micrococcus luteus* bacteria. The growth of *M. luteus* was inhibited around the *P. luminescens* spot, this inhibition resulting from ST antibiotic activity (41). Greater ST production by *P. luminescens* results in a larger inhibition zone. The diameter of the inhibition zone was 2.8 ± 0.1 (mean ± standard deviation) cm for the parent and 3.3 ± 0.1 cm for the mutant ($n = 2$), indicating that HcaE is not required for ST production.

Effect of *hcaE* disruption on PAL activity and *stlA* expression. The increase in the amount of extracellular endogenous CA resulting from *hcaE* disruption may be related only to a decrease in CA degradation, but it also may be the consequence of an increase in CA synthesis. Thus, the effect of *hcaE* disruption on PAL activity was investigated. Strains TT01R and TT01Δ*hcaE* were grown in LB; proteins were extracted at various times, and the PAL activity was measured. Interestingly, the PAL activity measured in crude bacterial extract was about threefold higher in the mutant than in the parent strain (Fig. 5A). The activity measured between 14 and 44 h of growth was 160 ± 70 pmol min^{-1} mg^{-1} in the TT01R extracts and 430 ± 100 pmol min^{-1} mg^{-1} in the TT01Δ*hcaE* extracts (Fig. 5A). An increase of PAL activity in the *hcaE* mutant may be due to an increase in the expression of the *stlA* gene, coding for the PAL enzyme. The *stlA* mRNA amount was measured

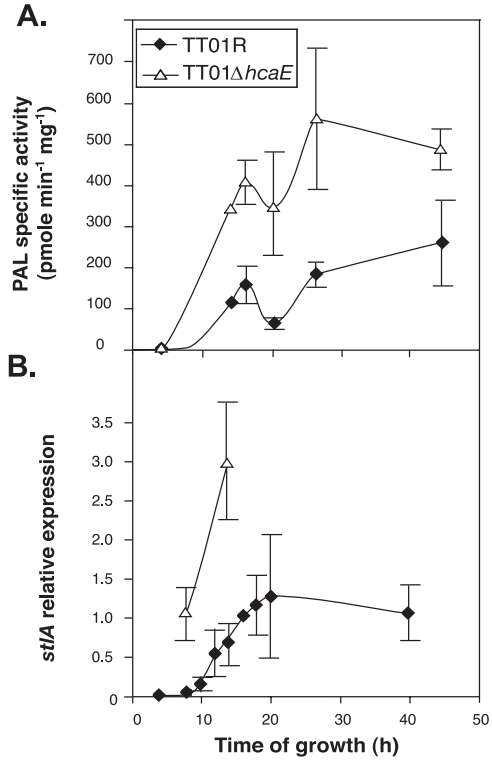

FIG. 5. CA synthesis by *P. luminescens*. The parent TT01R and the mutant TT01Δ*hcaE* strain were grown in LB. (A) Proteins were extracted at various times of growth, and PAL activity was measured. (B) Total RNA was extracted at various times of growth, and amount of *stlA* mRNA was measured by quantitative RT-PCR, using expression of *udp* for normalization. Expression levels of *stlA* in TT01R after 16 h of growth were arbitrarily fixed to 1. Measurements are expressed as the means ± standard deviation of the results of two independent experiments.

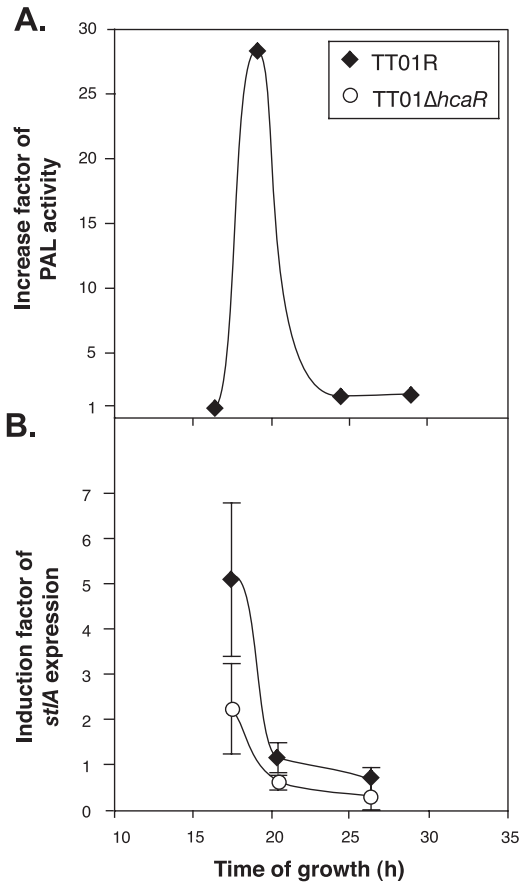

FIG. 6. Autoinduction of CA synthesis. (A) Effect of CA on PAL activity. TT01R was grown in LB with or without 1.3 mM CA. Proteins were extracted at various times, PAL activity was measured, and the increase factor was determined. The increase factor is expressed as the ratio of activity measured in the presence of exogenous CA over activity in the absence of exogenous CA. (B) Effect of CA on *stlA* expression. The parent strain TT01R and the mutant strain TT01Δ*hcaR* were grown in LB with or without 1.3 mM CA. Total RNA was extracted at various times of growth, and the amount of *stlA* mRNA was measured by quantitative RT-PCR, using the expression of *udp* for normalization. The induction factor is expressed as the ratio of the amount measured in the presence of exogenous CA over the amount in the absence of exogenous CA. Measurements are expressed as the means ± standard deviation of the results of two independent experiments.

by quantitative RT-PCR in total RNA extracted from strains TT01R and TT01Δ*hcaE* grown in LB medium. The expression of *stlA* was higher in the mutant than in the parent (Fig. 5B). This shows that *hcaE* disruption increases *stlA* expression, thus enhancing CA synthesis.

CA induces its own biosynthesis. The increase in PAL activity resulting from *hcaE* disruption may be the consequence of an increase in the amount of endogenous CA. Indeed, CA could induce its own synthesis similarly to other compounds, such as 2,4-diacetylphloroglucinol (an antibacterial phenolic compound) (35). The effect of CA on PAL activity was investigated. The wild-type strain TT01R was grown in LB in the presence or absence of 1.3 mM exogenous CA, and proteins were extracted at various times. The presence of exogenous CA strongly increased PAL activity—28-fold—after 19 h of growth (Fig. 6A). The increase occurred only after 19 h of growth, coinciding with the disappearance of exogenous CA from the extracellular medium (Fig. 3B). This increase may be related to the internalization of exogenous CA, which may start when CA begins to disappear from the extracellular medium.

This increase in PAL activity may be the consequence of inducing the expression of its structural gene, *stlA*. The amount of *stlA* mRNA was measured by quantitative RT-PCR in TT01R bacteria grown in LB in the presence or absence of 1.3 mM CA. After 17 h of growth, the amount of *stlA* mRNA was 5.1 ± 1.7 times greater in bacteria grown in the presence than in the absence of exogenous CA ($n = 2$) (Fig. 6B). After 20 and 26 h of growth, *stlA* expression was similar in bacteria grown with or without exogenous CA (Fig. 6B). Thus, the increase in PAL activity by CA was due to the induction of *stlA* expression.

Involvement of the HcaR regulator in CA-induced *hcaE* and *stlA* expression. In *E. coli*, CA induces the expression of the *hca* operon, and this induction requires the HcaR regulatory protein (12). The effect of CA on *hcaE* expression in *P. luminescens* was assessed by quantitative RT-PCR. In TT01R grown without exogenous CA, *hcaE* was expressed at the end of the exponential growth (Fig. 7). The presence of exogenous CA increased *hcaE* expression by 6.3 ± 2.5 times after 15 h (Fig. 7). Thus, CA induces the expression of the *hca* operon in *P. luminescens*. In the TT01Δ*hcaR* mutant bacteria, disrupted for *hcaR*, this induction did not occur (Fig. 7). Thus, in *P. luminescens*, HcaR is required for CA-induced *hcaE* expression during the stationary phase.

In *P. luminescens*, the *hca* operon encodes homologs of various enzymes that catalyze the first three steps of CA degradation (Fig. 1B and 2). plu2201 and plu2202 are located upstream of the *hcaR* gene and encode homologs of enzymes that catalyze the fourth and the fifth steps of CA degradation (Fig. 1B and 2). We investigated by quantitative RT-PCR whether plu2201 and plu2202 expression is induced by CA. In the parent TT01R strain, the expression patterns of plu2201 and plu2202 are similar to that of *hcaE* (Fig. 7). After 15 h of growth, plu2201 expression was 5.1 ± 0.8 times greater and plu2202 expression was 5.7 ± 0.1 times greater in the presence of exogenous CA than in its absence (Fig. 7). This showed that CA also induces plu2201 and plu2202 expression. No induction occurred in the TT01Δ*hcaR* mutant strain (Fig. 7). Therefore, the effect of CA on plu2201 and plu2202 expression requires HcaR.

CA induces the expression of the *hca* operon and the plu2201 and plu2202 genes. It also induces *stlA* expression. To know whether HcaR was required for this latter induction effect, *stlA* expression was measured in the TT01Δ*hcaR* mutant strain grown with or without 1.3 mM CA in LB medium. The presence of exogenous CA increased the amount of *stlA* mRNA 2.2 times in the TT01Δ*hcaR* mutant after 17 h of growth (Fig. 6B). This shows that HcaR is not required for CA-induced *stlA* expression.

DISCUSSION

Photorhabdus luminescens is one of the two known bacterial species able to synthesize CA from phenylalanine (Fig. 1A) (41). CA is the precursor for synthesizing ST, which has antibiotic activity (15, 32, 41). *P. luminescens* also produces ho-

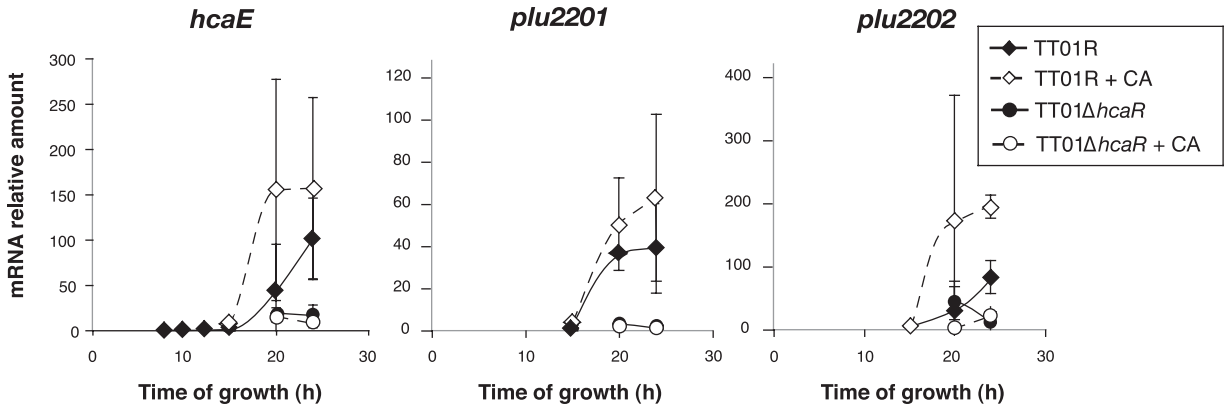

FIG. 7. Effect of CA on expression of *hcaE*, plu2201, and plu2202. The parent TT01R and the mutant TT01Δ*hcaR* strains were grown in LB with (dotted lines) or without (solid lines) 1.3 mM CA. Total RNA was extracted at different times of growth, and amounts of *hcaE*, plu2201, and plu2202 mRNA were measured by quantitative RT-PCR, using expression of *udp* for normalization. The expression level in TT01R bacteria grown for 15 h in the absence of exogenous CA was arbitrarily fixed at 1. Measurements are expressed as the means ± standard deviations of the results of two independent experiments.

mologs of the Hca enzymes catalyzing CA degradation in *E. coli* (Fig. 1B) (12). In this study, Hca enzymes from *P. luminescens* are shown to be involved in the degradation of endogenous CA, but not required for ST synthesis. CA was processed via at least two distinct pathways in *P. luminescens*.

P. luminescens releases CA at the end of exponential growth, with its concentration reaching 0.2 mM in the culture supernatant, and then rapidly degrades it at the beginning of stationary phase. The Hca dioxygenase is involved in this degradation. The first steps of lincomycin A biosynthesis in *Streptomyces lincolnensis* comprise the oxidation of the aromatic ring of tyrosine by dioxygenases (28). Thus, the Hca dioxygenase could catalyze the first step of the ST biosynthetic pathway in *P. luminescens*. However, HcaE, the alpha subunit of the Hca dioxygenase, was not required for ST production, strongly suggesting that the Hca dioxygenase is not required for ST synthesis. Besides, the ring-cleavage product of CA (compound III in Fig. 1) is probably not an intermediary compound of ST formation, strongly suggesting that the Hca enzymes are not involved in the ST pathway.

The maximal concentration of ST released by *P. luminescens* when grown in LB was 0.03 mM during the stationary phase (data not shown), while 0.2 mM CA was consumed. Thus, ST is a minority final product of CA processing. It means that either (1) CA is predominantly processed via the pathway involving the Hca enzymes or (2) ST is just an intermediate compound. The latter is plausible, as stilbene can react in many different ways, forming a variety of stilbene dimers and trimers or different stilbene glucosides in seemingly endless combinations (9, 24). The expression pattern of the *hca* operon coincided with the disappearance of CA from the culture supernatant. In addition, this disappearance was abolished by *hcaE* disruption. Together, these results suggest that the Hca dioxygenase is responsible for the majority of CA degradation.

In *E. coli*, the CA ring-cleavage product is eventually de-

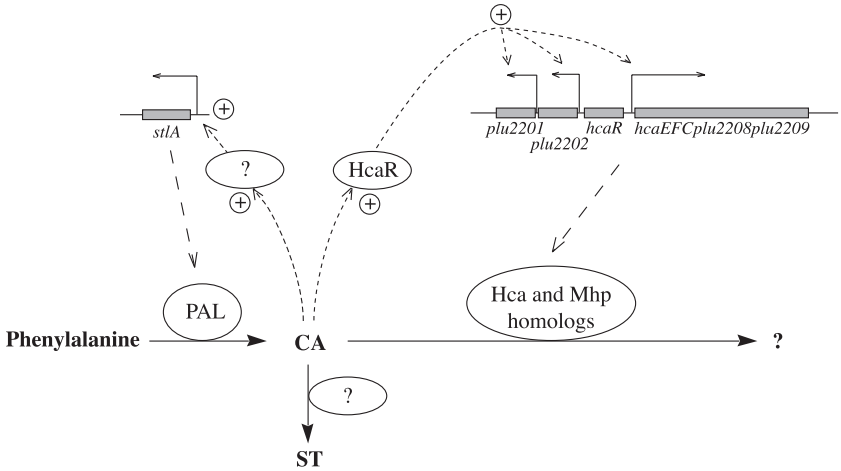

FIG. 8. Proposed model for CA utilization and response in *P. luminescens*. PAL converts phenylalanine to CA. CA is then processed via the ST biosynthetic pathway (encircled question mark) and via a catabolic pathway involving homologs of the Hca and Mhp enzymes. Final products (bold question mark) of the latter pathway are unknown. CA also induces its own synthesis by inducing the expression of *stlA*, coding for PAL via an unknown regulator (encircled question mark). CA also induces its catabolism by inducing the expression of the *hca* operon and the plu2201 and plu2202 genes, coding for the Hca and Mhp homologs. This induction requires the HcaR regulator.

graded into Krebs cycle intermediates by Mhp enzymes (12) (Fig. 1B). The *P. luminescens* homologs of *mhpC* and *mhpD*, plu2202 and plu2201, respectively, have an expression pattern similar to that of *hcaE*. Thus, the first five reactions of CA catabolism do occur in *P. luminescens*. No *mhpE* or *mhpF* homologs were found in the genome of *P. luminescens*, which is unable to use 3PP or CA as the sole carbon source. These observations suggest that the Krebs cycle intermediates pyruvate and acetyl-CoA are not the final products of CA degradation in *P. luminescens*. The role of the Hca enzymes in the *P. luminescens* life cycle remains to be understood. The Hca dioxygenase is not involved in pathogenicity, as the disruption of *hcaE* does not affect *P. luminescens* virulence in *Bombyx mori* larvae (8). A *P. luminescens* mutant disrupted for *stlA*, which does not produce CA, has a delayed virulence in *Manduca sexta* larvae (15). However, this delay was shown to be due to the absence of ST production, and not an absence of CA production (15).

CA induces the expression of genes involved in its synthesis (*stlA*) via an unknown regulator and in its degradation (the *hca* operon, plu2201, and plu2202) via the HcaR regulatory protein. CA synthesis self-induction may explain why the disruption of *hcaE* increases *stlA* expression. The disruption of *hcaE* increased the amount of intracellular CA (data not shown), which may in turn increase *stlA* expression, leading to increases in PAL activity and ST production. Other examples of autoinduction in bacteria have been reported, such as the behavior of siderophore pyochelin in *Pseudomonas aeruginosa* (31) or yersiniabactin in *Yersinia enterocolitica* (29). The best-known autoinducers are quorum-sensing molecules, like *N*-acyl-homoserine lactones (16). In *P. luminescens*, the regulation of the CA level shares common features with that of a quorum-sensing molecule, autoinducer 2 (AI-2). *E. coli* and *S. enterica* serovar Typhimurium release AI-2 into the medium at the end of exponential growth and then internalize it at the start of the stationary phase, making AI-2 disappear from the culture supernatant (37, 42). After being internalized, AI-2 is converted to currently uncharacterized products. A similar behavior for CA in *P. luminescens* is reported here. Besides, both AI-2 and CA are small molecules with similar molecular sizes (150 and 148 g mol^{-1}, respectively), and both are able to activate genetic expression. Therefore, it should be interesting to investigate whether CA itself is a quorum-sensing molecule.

In conclusion, in *P. luminescens*, CA induces its own synthesis, is actively released into the medium at the end of exponential growth, and is then itself consumed by at least two pathways: the ST biosynthetic pathway and a pathway involving the Hca enzymes, yet to be characterized (Fig. 8). The parallels established between AI-2 and CA may provide some direction to future studies.

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